THE ROLE OF SPECIFIC SURFACE ANTIGENS IN CELL ADHESION. PART I. THE REAGGREGATION OF SPONGE CELLS [1]

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In the "auto-antibody concept" of Tyler (1940, 1942, 1946, 1947) and the "molecular ecology" of Weiss (1941, 1947, 1950) one important part is the hypothesis that contiguous cell surfaces are normally held together, at least partly, by forces like those between antigens and homologous antibodies. The forces are assumed to be associated with specific macromolecules, of at least two stereochemically reciprocal types per cell, so held in the cell surface that they can combine from cell to cell. The hypothesis has been shown to be compatible with a diverse array of observational and experimental data, particularly by Tyler (1947) and Weiss (1947). However, both workers have emphasized the need for new experiments. "It is obviously essential for us first to obtain further evidence for or against the existence of such antigen-antibody like systems of complementary substances within cells" (Tyler, 1947; p. 17). "The subtle means by which cells can recognize each other and their appropriate environments are still wholly conjectural" (Weiss, 1950; p. 184).

It has been proposed (Tyler, 1947) that for further investigations along this line, the phenomena of selective reaggregation of dissociated sponges (Wilson, 1910; Galtsoff, 1925) and of the segregation of cells that occurs in certain combinations of embryonic tissues (Holtfreter, 1939) should constitute favorable material.

One type of experiment is suggested by the proposal that *macromolecules* are involved. Such molecules might act as antigens when injected from one species into another. Antisera so produced should affect, in a predictable way, processes involved in cell adhesion. Experiments of this type are reported here on dissociated *Microciona prolifera* and *Cliona celata*. In a second paper (Spiegel, 1954) experiments on embryonic cells of *Rana pipiens* and *Triton alpestris* are reported.

NORMAL REAGGREGATION AND SEGREGATION

A review of the essential phenomena in sponge reaggregation and segregation may be a useful preliminary to consideration of the experimental results.

Wilson (1907) first showed that a sponge could be dissociated, by pressing through bolting cloth, into isolated cells which would form a number of reaggregates within a day's time. Galtsoff (1925) showed that clusters of about 2,000 or more cells are capable of reorganizing, after 5–6 days under proper conditions, into

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perfect miniature sponges. Various aspects of the phenomena have been studied by Wilson (1910, 1932), Galtsoff (1923, 1925, 1926, 1929), de Laubenfels (1927, 1932) and Brøndsted (1936) among others, and the following account is based partly on their observations and those of the present author.

After disaggregation the cells slowly settle down. Upon coming in contact with the substratum, archeocytes and pinacocytes flatten out, adhere and put forth hyaline pseudopodia. They begin to move almost immediately. The archeocytes are more active and move at about 0.6–3.5 microns per minute, changing direction apparently at random roughly three times per hour. Under extremely favorable conditions their velocity may reach 20 microns per minute (Galtsoff, 1925). Movement continues for about 24 hours.

Upon coming in contact with one another, the cells generally coalesce. The plasma membranes of the apposed cells seem to join together zipper-wise, proceeding from the area of first contact. After 5–10 minutes, the surface of union between the two cells is indistinct or has vanished and it appears that the clear outer hyaloplasm has formed a common matrix for the two inner granuloplasms which remain distinct and separate from one another.

As new contacts continue to occur, larger and larger aggregates are formed, the number and size depending on the density of the original suspension and on temperature, osmotic pressure, ionic composition, pH of the medium, etc. Aggregates increase in size by addition of single cells or by coalescence with other aggregates. Under Galtsoff's standard conditions some aggregates were about 0.5 mm. in diameter, consisting of roughly 2000 cells, after one day. Neither number nor size of aggregates changes further after ameboid movement stops at the end of 24 hours.

Present observations reveal certain additional features of interest. *Movements of granules during cell coalescence.* In Microciona while a pair of cells is coalescing, their granules undergo a series of intense movements and shifts in position, resulting in a striking reorganization of the granuloplasm. The granules which seem originally to be distributed at random in the cytoplasm of an isolated cell reorient so as to lie immediately inside the surface of the granuloplasm. The archeocyte nuclei, which previously had been partly or wholly obscured by the granules, are now quite evident with conspicuous light green nucleoli. *Fusion of hyaloplasm in cell coalescence.* An aggregate of two cells is able to put out a pseudopodium about 15 microns long which is approximately twice that formed by an isolated cell. An aggregate of three cells may form pseudopodia ranging from 30 to 35 microns in length. Large aggregates of 100–200 cells occasionally put out pseudopodia 200–300 microns long, of quite striking appearance. In general pseudopodium size varies with about the 1.2 power of cell number. The hyaloplasm of all the cells evidently acts in some sense as a unit, freely available to the aggregate's single pseudopodium. The microscopic appearance during coalescence suggests that the interface between the hyaloplasms of two coalescing cells disappears.

EFFECTS OF ANTISERA ON REAGGREGATION AND SEGREGATION

Materials and Methods

Specimens of *Microciona prolifera* (the large encrusting red sponge) and *Cliona celata* (the yellow sulfur sponge) were employed. The colonies were kept in running sea water in the experiments done at the Marine Biological Laboratory. For

work at the University of Rochester, living colonies were shipped from Woods Hole by Air Express and kept in sea water, replaced at two-day intervals, at a temperature of 1–2° C. Experimental results were similar under the two maintenance methods.

Quantitative studies of reaggregation. For the experiments, it seemed desirable to have a quantitative expression of the extent of reaggregation at successive times. The following method was used.

For each series of observations a fragment of fresh sponge was carefully dried with filter paper and a one-gram portion squeezed through bolting cloth into 40 ml. of sea water. Ten ml. of the suspension were transferred to a syracuse dish and examined under the microscope at 430 × magnification. At intervals the aggregates were counted in each of five microscope fields taken at random, and the values were averaged. For these counts "aggregate" was defined as any coalesced group of *four or more cells*, and it is in this sense that the word will be used hereafter. Four cells constitute the smallest grouping that can be readily recognized as an aggregate as distinct from cells in contact, in routine examinations.

Typical results over a 24-hour period are shown in Figure 1. Such a figure will be called a reaggregation curve and each of its ordinates, an aggregate count.

The curve is satisfactorily reproducible under standard conditions; as an example, seven unselected curves are shown in Figure 2. Up to four hours the extreme variation of counts at any one time is about two aggregates. Between four and eight hours counts are somewhat more variable. But they are quite uniform again at 24 hours, at least under normal conditions: 12 such counts, unselected, range from 3.9 to 4.5. The progress of reaggregation as illustrated by these curves

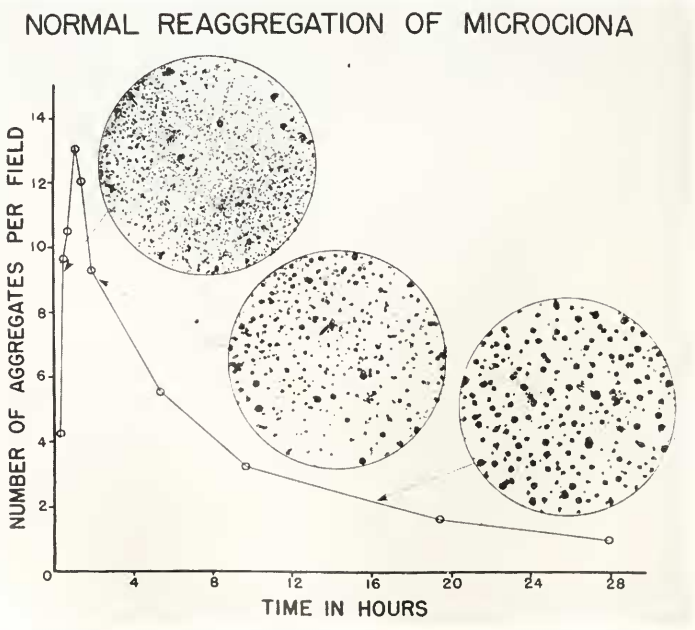


FIGURE 1. Reaggregation of *Microciona prolifera* in sea water.

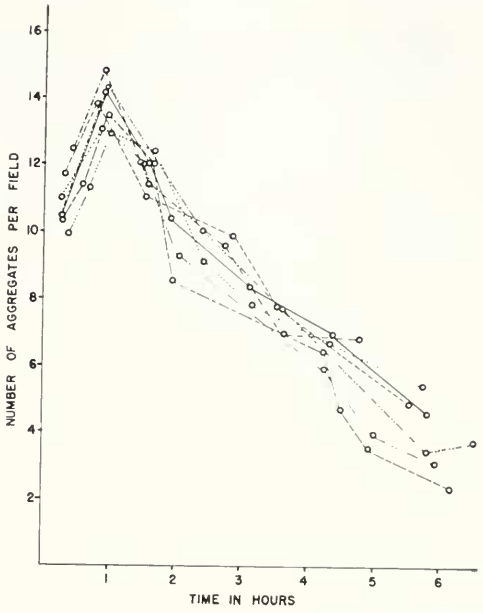


FIGURE 2. Reaggregation of *Microciona prolifera* in normal serum; seven unselected curves demonstrating reproducibility under standard conditions. Essentially the same curve is obtained in sea water.

shows an initial increase in number of aggregates (of small size) to a maximum at about one hour, followed by a decrease in number (fusion of aggregates with corresponding increase in size).

In a mixed suspension of the red-colored Microciona and the yellow Cliona cells, 24 hours after dispersal, the aggregates are observed to be species-specific, as reported by Wilson (1910), Galtsoff (1925) and de Laubenfels (1927). A few cells of one type in an aggregate prevailingly of the other species cannot readily be distinguished. If such admixture occurs, its extent is too slight to be readily detectable.

The Microciona aggregates formed in a mixed suspension are more numerous and correspondingly smaller than in a pure Microciona suspension. They are, however, normally well rounded with a well-defined hyaline membrane. Similarly, the Cliona aggregates, more and smaller than in pure Cliona preparations, have the characteristically jagged contour but easily seen hyaline coat of pure Cliona suspensions. Occasionally an aggregate of Microciona and one of Cliona are observed in close contact, but their hyaloplasms remain separate and the two fail to adhere.

It is important, for interpreting the experimental results, to note here that the condition seen at 24 hours is preceded by an initial temporary intermixture of the two types of cells. But at 4–8 hours, after dissociation, in most aggregates islands of cells of one species are seen in a matrix of the other species; they are apparently separating from it.

The smaller size of aggregates in mixed suspensions is perhaps partly a consequence of the sorting out: contacts which would lead to a permanent increase of

aggregate size in a pure suspension are cancelled out in a mixed preparation by the later sorting out where interspecific contacts had occurred. To some extent each species may also interfere just mechanically with the other's aggregation as Galtsoff (1925) has shown glass particles and starch grains to do.

Preparation of stock suspensions. For each of these a piece of fresh sponge was cut into fragments 2–3 mm. across, which were carefully dried on filter paper. A known weight of fragments was squeezed through a small bag made of bolting silk, mesh 20, into sea water. Six types of suspensions were used, and freshly prepared before each use; they were as follows, M representing Microciona, and C, Cliona:

Stock suspension	1	2	3	4	5	6
			6.2M			1 M
Sponge (g.)	6.2M	6.2C	6.2C	1M	6.2C	6.2C
Sea water (ml.)	20	20	40	40	40	40

Suspensions 1, 2 and 3 were used in preparations of antisera; their composition was so chosen as to give the same total nitrogen per ml.; 1.3 mg. in each, by micro-Kjeldahl determination. Suspensions 4, 5 and 6 were used in tests of antiserum effects; their composition was so chosen as to give about equal initial numbers of free cells per microscope field in 4 and 5.

Preparation of antisera. For most of the experiments the antisera were derived from nine New Zealand giant rabbits, three for each suspension. Nine subcutaneous injections of 0.1 ml. of stock suspension, were given over a period of three weeks.

At 8 days after the last injection blood was collected in sterile 50-ml. Pyrex centrifuge tubes, allowed to clot at room temperature, kept at 40° C. for one hour, the clot loosened, and the blood centrifuged at 350 G for 15 minutes. The clear straw-colored serum was pipetted off and the following were added per 50 ml. serum: 0.2 mg. Penicillin G potassium, 0.2 mg. Streptomycin calcium chloride complex and 0.2 mg. Aureomycin. The mixture was kept in sterile serum bottles at 1° C. until use.

Serum obtained from animals injected with Microciona cells will be referred to hereafter as anti-Microciona serum; others will be similarly called anti-Cliona and anti-Microciona : Cliona.

Test of antiserum titers. These were done to determine, by standard methods, that the sera actually contained antibodies to sponge antigens. A 6.2-gram portion of Microciona was homogenized for five minutes in a Ten Broeck glass homogenizer with 20 ml. of 0.01 *M* phosphate buffer at pH 7.2 and containing 0.85% NaCl. The homogenate was centrifuged at 485 G for 20 minutes and the supernatant collected. The same procedure was carried out with 6.2 grams of Cliona cells in 20 ml. of buffered saline and with 6.2 grams Microciona plus 6.2 grams Cliona in 40 ml. of buffered saline.

Precipitation tests were set up by layering 0.5 ml. of undiluted extract and of five 10-fold dilutions (1:10 to 1:100,000 in buffered saline) over 0.5 ml. of homologous, undiluted antiserum, or over saline or normal serum as controls in 10 × 75 mm. serological tubes. All tubes were incubated at 37° C. for one hour and then

examined for presence of a precipitate ring at the interface. All nine sera produced rings of antigen-antibody complex at all antigen dilutions down through 1:10,000. Controls were negative. The tests show, then, the presence of antibodies to sponge antigens extractible in saline, although these do not necessarily mean that cell surface antigens are involved.

Tests of antiserum effects on reaggregation. Three ml. of an antiserum were mixed with an equal volume of double-strength van't Hoff solution alpha (58.44 grams NaCl, 1.62 grams KCl, 2.24 grams $CaCl_2$, 16.18 grams $MgCl_2.6H_2O$, 9.38 grams $MgSO_4.7H_2O$, distilled water to one liter mark), producing a medium approximately isosmotic with sea water. Four ml. of suspension 4, 5 or 6, freshly prepared, were added. Controls were 4-ml. portions of the same cell suspension added to three ml. of double-strength van't Hoff solution plus three ml. of serum from a normal uninjected rabbit (18 experiments), or to six ml. of ordinary strength van't Hoff (four experiments). In five experiments there were no simultaneous controls.

Altogether 63 antiserum and 22 control preparations were observed in 27 experiments; normal reaggregation was also followed in 13 other preparations, 10 in normal serum and three in van't Hoff solution.

Results

Microciona in anti-Microciona serum

Reaggregation curves for Microciona cells during the first three hours in normal serum and in two anti-Microciona sera are shown in Figure 3 with photomicrographs at 2.75 hours. In two other antiserum preparations studied by reaggregation curves, results were obtained scarcely distinguishable from the two pictured here.

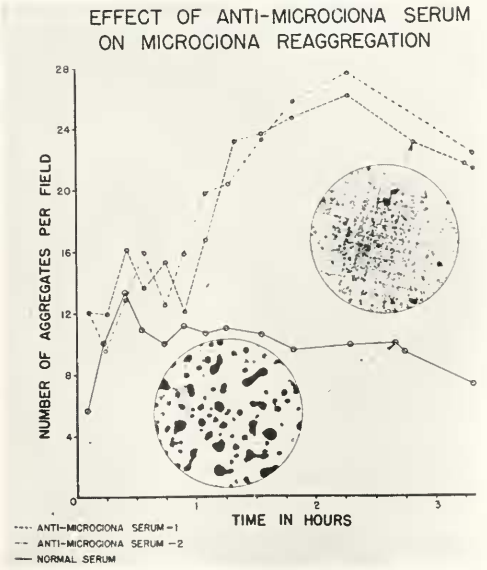


Figure 3. Effect of anti-Microciona serum on Microciona reaggregation.

3. contact of some "cells" with the substratum, and adhesion, during preliminary swirling;

4. settling of initially suspended "cells" throughout the first 2 hours;

5. contact and coalescence among cells and aggregates creeping on the substratum.

A reaggregation curve during the first few hours reflects these processes jointly. But 1, 3, and 4 are really irrelevant to the phenomena of primary interest here. Furthermore the Microciona experiments in anti-Microciona serum showed that processes 2 and 5 operate alike, so that no significant information is obtained within the first few hours that could not be more easily gotten from any single later observation. Since the same would presumably be true for Cliona and for other antisera, attention was confined in subsequent experiments to observations at 24 hours after dissociation (when cell movement ceases and no further coalescence occurs).

Other combinations of sponge and antiserum

Microciona (*Fig. 4*). In anti-Cliona serum there were no signs that reaggregation had been inhibited. The aggregates in anti-Cliona serum were as large or larger than normal (Fig. 4). Five experiments gave consistent results, the aggregates counts at 24 hours averaging about 4.3.

In anti-Microciona:Cliona serum, Microciona formed aggregates of number and size intermediate between those in normal serum and in anti-Microciona serum; reaggregation was partly inhibited.

Cliona. There were more, and smaller, aggregates in anti-Cliona serum than in normal serum, indicating an inhibition of reaggregation analogous to that of Microciona in anti-Microciona serum.

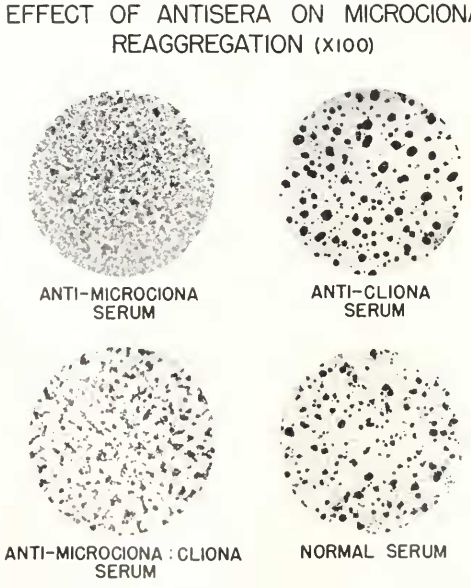


FIGURE 4. Reaggregation of Microciona in antisera.

In anti-Microciona:Cliona serum, there were fewer aggregates than in anti-Cliona serum but more, and smaller, than in normal serum. The inhibition was partial like that of Microciona in the same serum.

With anti-Microciona serum, there were fewer and larger aggregates than in anti-Cliona serum but smaller than in normal serum, and scarcely larger than in anti-Microciona:Cliona serum.

Microciona-Cliona mixtures. In normal serum the initially mixed cells sorted out. The Microciona aggregates were smaller than are formed by pure Microciona as were also the Cliona aggregates. The probable reason for this has already been suggested above and has nothing to do with their surface antigens.

In anti-Cliona, as in normal, serum, there were no mixed aggregates and the Microciona aggregates were normal, though smaller than formed by Microciona alone in normal or in anti-Cliona serum. The Cliona components of the suspension were much like Cliona in anti-Cliona. The results are what would be expected if anti-Cliona serum contained antibodies to surface antigens of Cliona but not Microciona.

In anti-Microciona serum, the mixed suspension did not form any large aggregates of the Microciona type, but many isolated cells and small clusters were seen. The larger aggregates were found to consist of Cliona cells. Most of them were about as large as formed by Cliona alone in anti-Microciona serum. They were smaller, though, than those of Cliona alone in normal serum. This can be interpreted as a consequence of the cancelling of previous associations by ejections of Microciona cells from mixed clusters as suggested above for mixed cells in normal serum.

In anti-Microciona:Cliona serum, a result was obtained diametrically opposite to the effect of the other antisera on pure or mixed suspensions. There were large aggregates of general shape intermediate between Microciona in normal serum and Cliona in normal serum. Many of the aggregates were much larger than in any other cell-serum combination. At the time of the experiment it was noted that (1) cells, or cell groups, of the 2 species were intermingled apparently at random throughout the aggregates: at least there were no patches of red Microciona cells or of colorless Cliona cells, but the aggregates were of a uniform, intermediate color; (2) the aggregates had a somewhat porous structure; in the reticulum between pores, the cell-to-cell contacts seemed to be about as close as in either Cliona or Microciona in normal serum.

EFFECT OF CALCIUM ON REAGGREGATION

Galtsoff (1925) and de Laubenfels (1932) have reported that reaggregation is impeded in media outside a limited range of calcium concentrations. But Agrell (1951) was unable to maintain *Halichondria panicea* suspensions as isolated cells in citrate or oxalate solutions. In view of this inconsistency, and considering the apparent role of surface antigens, it seemed desirable to check some of the previous work on the action of calcium. Three types of experiments were carried out: in calcium-free media, in the presence of a calcium-complexing agent, and in high calcium solutions.

Effects of calcium-free media. Galtsoff (1925) reported that in NaCl or KCl solutions, the cells do not move and no coalescence occurs, and that even after 24 hours, the substratum is covered with single cells which die about a day later.

Following Galtsoff's methods a piece of Microciona weighing two grams was squeezed through bolting cloth into 20 ml. of 0.52 *M* NaCl (isotonic). The suspension was centrifuged at 62 G for five minutes and the supernatant drawn off with a pipette; fresh solution was added and the tube containing the cells vigorously shaken. The centrifuging and subsequent washing were repeated three times. Two ml. of this suspension were added to (1) eight ml. of 0.52 *M* NaCl, to (2) eight ml. of 0.52 *M* KCl, and to (3) eight ml. of normal sea water, in syracuse dishes. After 24 hours, the number of aggregates, per microscope field, was counted for five random fields.

Medium	1	2	3
Average no. of aggregates per field	0	0	4.2

At 24 hours, the NaCl and KCl cell suspensions were swirled and large macroscopic aggregates were formed. Identical results were obtained if the cells were suspended for 24 hours in an isotonic NaCl solution and then washed with subsequent centrifuging, three times, re-suspending each time by vigorous shaking. It is clear, therefore, that the cells fail to reaggregate not because they were dead or lacked adhesiveness after 24 hours, but because they had lost the ability to move.

Effects of Versene on reaggregation. It might be argued the NaCl or KCl solutions do not completely deprive the cells of calcium, and in order to check the above observations, it was desirous to find some means of effectively binding calcium specifically, so that it would no longer be available to the cell. Sodium oxalate or sodium citrate can combine with calcium to form an insoluble precipitate but this might disrupt the internal organization of cells and consequently bring about their death. It was necessary, therefore, to use a substance which is capable of forming a strong soluble complex with calcium and which is not toxic to living organisms. Versene (ethylenediamine tetra acetic acid) is such a compound; at pH 7.0 it sequesters calcium from solutions in a 1:1 molar ratio, by chelation.

Two grams of Microciona were squeezed through bolting cloth into 20 ml. of 0.05 *M* di-sodium versenate in 0.50 *M* NaCl, 0.01 *M* phosphate buffered at pH 7.00. The suspension was centrifuged at 62 G for five minutes and the supernatant removed with a pipette; fresh solution was added and the tube containing the cells vigorously shaken. The centrifuging with subsequent washing was repeated three times. It was calculated that all of the calcium and possibly some magnesium ions should at that point have been chelated with Versene. Two ml. of the suspension were then added to (1) eight ml. of the di-sodium versenate solution and to (2) eight ml. of normal sea water, in syracuse dishes. After 24 hours the dishes were examined under the microscope and the aggregates counted.

The same results as in NaCl and KCl were obtained.

Medium	1	2
Average no. of aggregates per field	0	4.5

At this time the bottom of the versenate dish was covered with single isolated cells which did not exhibit either ameboid movement or coalescence. When the dish was swirled large macroscopic aggregates were formed. Identical results were obtained with cells first suspended for 24 hours in di-sodium versenate and then centrifuged followed by washing three times, re-suspending each time by vigorous shaking. Since a normal aggregate count was obtained with cells trans-

ferred to sea water, it is readily apparent that the cells remained alive in the versenate solution.

The above experiments offer additional evidence to support the conclusion that calcium deficiency prevents reaggregation by the inhibition of ameboid movement and does not affect or alter the adhesiveness of the cells to each other.

Effects of calcium excess on reaggregation. Although no role of calcium in cell adhesion is indicated by the effects of calcium deficiency, one might be detected by the effects of calcium excess. Therefore, one gram of Microciona was dissociated into 40 ml. of normal sea water and two ml. of the suspension were added to eight ml. of normal sea water, and to seven ml. normal sea water plus one ml. of 0.35 *M* $CaCl_2$ in syracuse dishes.

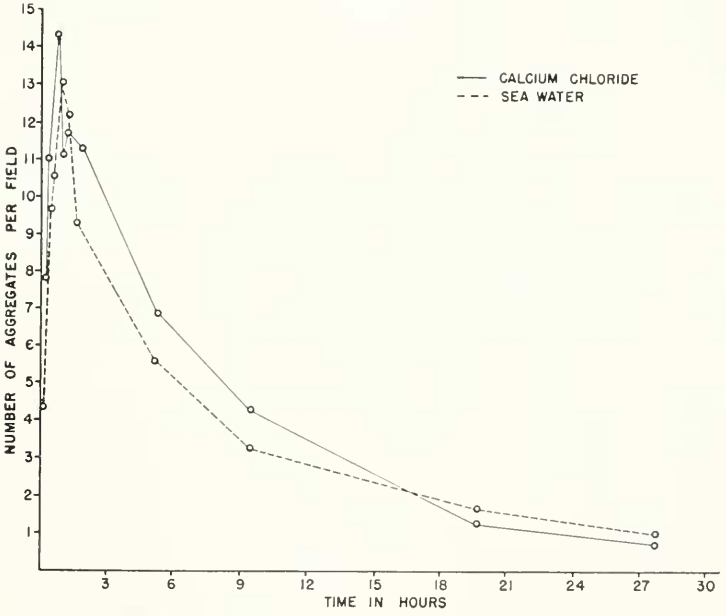


FIGURE 5. Reaggregation of Microciona in normal and high calcium media.

Aggregate counts were made at varying intervals; the reaggregation curves are shown in Figure 5. Differences are well within the limits of variation among preparations in sea water.

In experiments under identical conditions, Galtsoff (1925) found 7.1 times as many aggregates (meaning less reaggregation) in excess-calcium solution as in normal sea water at 24 hours. The difference in results is difficult to explain; it leaves considerable doubt whether reaggregation is significantly affected by increasing the calcium of the medium 4.1 times.

DISCUSSION

The present results show that reaggregation is inhibited by the presence of homologous antibodies. The usual expectation, when a specific antiserum is added to a suspension of the homologous cells, is that an agglutination of the cells will

occur. The first point to be discussed, then, is why such agglutination by antibodies does not appear in the present experiments. There are at least three conditions under which agglutination may not occur even though specific reaction takes place between surface antigens and homologous antibodies:

(1) *Extreme antibody excess.* Under this condition the cells are, presumably, completely saturated with agglutinin before cell-to-cell contacts are made. The cells are incapable of reacting with each other since all their receptor sites are occupied. Such a hypothesis has been offered by McKerns and Denstedt (1950) to account for the invariable persistence of unagglutinated red blood cells in agglutinated samples even when potent isoagglutinating serum is used. Failure of agglutination in antibody excess is, of course, the expectation from the Marrack-Heidelberger-Pauling "mutual multivalence" hypothesis (see Boyd, 1947 for ref.). It does not seem likely, however, that the sera used in the present investigation are of such high titer that the vast majority of cells are saturated before cell collisions take place.

(2) *Univalent antibodies.* In this case it would be assumed that the antibodies formed by a rabbit in response to injections of sponge antigen are chiefly of the univalent type. These antibodies could combine with surface antigens but since each molecule of antibody possesses only a single group reactive with antigen no agglutination takes place. This type of antibody seems to be commonly formed in Rh antisera (Wiener, 1944) and other immune antisera (see refs. in Tyler, 1945). Such a hypothesis is adequate for explaining inhibition of sponge reaggregation by homologous antiserum but offers no explanation for the apparent agglutination of mixed suspensions of cells by the homologous antiserum.

(3) *Structure of cell surface.* Professor Albert Tyler (personal communication) has suggested a third condition under which agglutination may not occur. One may assume that the surface of the sponge cell is a folded, and probably also flexible, structure. Most of the surface antigens would then be so situated that the two or more valence groups of a single antibody molecule would tend to react with antigen on the same cell. Since the surface of a sponge cell is observed to change its shape, as evidenced by pseudopodium formation, it seems reasonable to assume that there is enough flexibility and movement so that the valence groups of the antibody molecule would be able to contact two or more of the receptor sites on the same cell surface. Very few, if any, valence groups would then be available for reaction with receptor sites on other cells. This, then, can account for absence of agglutination by homologous antiserum and the inhibition of reaggregation. It can also account for the clumping obtained with mixed cells (of the two species of sponge) in antiserum vs. mixed cells. Here it is assumed that heterovalent antibodies are formed. Considering (for simplicity) only two valences on the antibody molecule, one may be termed anti-M, the other anti-C. Such antibody molecules would react at one end with cells of one species of the mixed suspension (say M cells) leaving a valence group (anti-C) free to contact the cells of the other species.

The failure of ostensibly multivalent antibodies to cause agglutination has been noted in several immunological systems. Gleeson-White *et al.* (1950) and Coombs *et al.* (1951) have studied the reactions of ox red cells in rabbit or guinea-pig anti-ox sera. The red cells of most oxen fail to agglutinate in such sera. Coombs *et al.* suggest that the failure is due to a deep location of the antigens so that a single

antibody molecule, even if multivalent, after combining with one cell cannot reach receptors of a second cell.

Coombs *et al.* have been able to accomplish the agglutination by certain devices which, in effect, can be considered to supply links between the antibody molecules attached to the different cells. For example, anti-rabbit globulin serum (produced in goats) will agglutinate the ox cells that have been treated with the rabbit anti-ox cells antiserum. This is particularly effective when the procedure is a serial addition of anti-globulin, then globulin, then anti-globulin, which is pictured as forming a longer chain, and provides strong support for the hypothesis of "deeply located" antigens.

This hypothesis does not, however, enable us to account for the agglutination of mixed cells by anti-mixed cells serum. It was therefore modified as described above in terms of interaction of a single antibody molecule with different parts of the same cell surface. Apart from the validity of the interpretation it appears clear from the work of Coombs *et al.* that there can be failure of agglutination, even in presence of multivalent antibody, with certain types of cells.

The effects of antisera on sponge reaggregation in relation to the Tyler-Weiss hypothesis will be considered in a stepwise discussion of the results.

1. *Anti-Microciona serum inhibits reaggregation of dissociated Microciona.* This conclusion can scarcely be doubted from the joint evidence of reaggregation curves up to three hours in standard and alcohol-killed suspensions, aggregate counts at 24 hours, microscopic appearance and size of aggregates at 2.75 and 24 hours, in normal serum and antiserum.

2. *The primary inhibiting effect of antiserum is on the process of adhesion.* The initial step in reaggregation, ameboid movement producing random contacts between cells, is normal in antiserum. But even when cells make contact in antiserum they fail to coalesce. There is no implication here of the mechanism by which the area of contact increases such as was suggested by Schmitt (1941).

3. *Some substance or class of substances "in" the cell surface is essential to the process of adhesion.* This conclusion is based on three considerations. (i) The zipper action, by which two impinging cells increase their area of contact, leads into the persisting contacts between cell surfaces within an aggregate. (ii) It seems probable that the same forces are responsible for the initial adhesive and the permanent contacts. The probability of this proposition is difficult to estimate for sponges because the boundary which can be seen, while two cells are joining, disappears within a few minutes. It is this change to which Galtsoff (1925) applied the term coalescence. Apparently the two hyaloplasms merge and the factors responsible for binding the two cells together then become simply part of the structure of the hyaloplasm. (iii) The *intrinsic* factors that hold cells together are commonly thought to be in the cell surface, *e.g.*, lipids. For many types of cells, extrinsic factors have been shown to be equally important, *e.g.*, calcium in salt linkage between lipids or proteins of adjacent cell surfaces, or "intercellular cement" which may involve a similar chemical bounding of an extrinsic substance to surface materials of two cells (Chambers, 1940). Other possible extrinsic factors are materials such as histones (Schmitt, 1941).

4. From steps 2 and 3, *the primary effect of antiserum is on some substance or substances in the cell surface.* Adhesions of two cells must involve interactions of their surface. It seems extremely unlikely that the antibodies affecting adhesion

would enter the cell producing some change inside which is later manifested in altered properties of the surface. A direct action of antibodies on the surface is more reasonable and can explain the results.

5. From the steps 1 and 4 it is reasonably probable that *anti-Microciona serum blocks the normal action of the materials in the cell surface that are essential for adhesion.*

6. *The only substances in anti-Microciona serum that are not also present in normal serum are antibodies to substances of Microciona that can act as antigens.* This has a three-fold basis. (i) Three anti-Microciona sera gave positive precipitin tests with Microciona extracts; two other anti-Microciona sera had the same effects on reaggregation as the three with precipitin tests. Therefore all five almost certainly contained antibodies. (ii) Three normal sera gave negative precipitin tests with Microciona extracts; two others, not precipitin tested, had no effect on reaggregation nor did the three tested. Therefore all five almost certainly had no antibodies to Microciona. (iii) Antisera do not normally differ qualitatively from normal sera, where comparable animals are used, except by presence of antibodies (Boyd, 1947).

7. From steps 5 and 6, *the normal role of certain surface antigens in adhesion is blocked by homologous antibodies present in antisera to Microciona cells.* The antigens will be represented by the symbol M, and the antibodies by anti*M*.

8. From comparable, less extensive tests, and by similar reasoning, *antigens A, in the surface of Cliona and essential in adhesion, are blocked in function by antiA in anti-Cliona serum.*

9. *M and A are specific antigens.* The main evidence here is that anti-Cliona serum does not block reaggregation of Microciona; the reasoning is analogous to steps 1–7. With Cliona in anti-Microciona serum there is some inhibition of reaggregation. One possible explanation for this is that Microciona possesses below the cell surface an antigen immunologically similar to a surface antigen of Cliona.

10. *M is inactive with respect to adhesion because it is combined with antiM.* This follows from step 7 and from the general knowledge that the only significant action of antibodies, under circumstances analogous to the present, is to combine with their homologous antigens (Boyd, 1947). The compound will be represented as M-anti*M*, without any implication as to the actual number of molecules of either M or anti*M* in the compound.

11. *M is not completely removed from a blocked cell.* This must be concluded from the fact that reaggregation can occur after washing an antiserum-treated suspension in sea water. It does not seem likely that such washing dissociates an antigen-antibody complex since such dissociation evidently involves more drastic treatment, such as high salinity (Heidelberger and Kabat, 1937) or high pH (Liu and Wu, 1938; Sternberger and Pressman, 1950). A more likely interpretation is that M extends below the surface; perhaps the entire hyaline layer is a gel of M and that M, at the surface, normally goes slowly into solution in sea water and is continuously replaced by M manufactured below. On this basis then, M-anti*M* would leave the surface slowly, or more rapidly by washing the cells, which would then be capable of reaggregating.

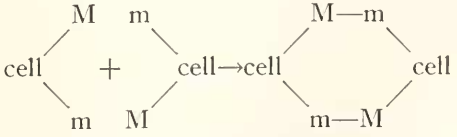
12. *The region of the M molecule to which antiM attaches is either* (a) *the site that is involved in adhesion, or* (b) *near enough to that site so that antiM overhangs or somehow interferes with the activity of the adhesion site.*

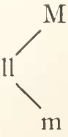
This is based on step 11 and 3 other considerations. (i) There seem to be only two possible ways of operation of M in normal adhesion: either through an extrinsic binder like calcium, *e.g.*, cell-M-Ca-M-cell; or by some type of direct bonding between M of one cell and M or some other substance m of another cell, *e.g.*, cell-M-m-cell, as envisaged by Tyler and Weiss. In this case a single cell can be assumed to have both M and m on the surface. (ii) The former seems unlikely considering particularly the failure of Versene to prevent reaggregation and the apparent absence in Microciona of anything able to act as an intercellular cement. De Laubenfels (1932) cites some evidence suggesting that, in dissociated *Iotrochota birotulata*, an intercellular slime is dispersed throughout the medium and brings cells or cell groups together, presumably by some sort of contraction. The writer has not seen, or had evidence of, any such material in Microciona. When a suspension is stirred the cells or cell groups move independently of each other; there are no signs of intercellular traction, nor any visible strands that could be identified as slime. (iii) Conclusion 12 follows from the remaining possibility: that the process of adhesion is essentially cell-M + m-cell → cell-M-m-cell.

13. *Alternatives a and b of step 12 cannot be readily distinguished.* The antibodies might well be directed against some constituent of the surface that is not directly concerned in reaggregation. Such bound antibodies might partly overlie the chemical sites involved in adhesion. This possibility cannot be distinguished on the basis of present evidence from the possibility that the antibodies combine directly with the chemical sites involved in adhesion.

14. It is likely that *adhesion depends on the presence, in the cell membranes, of two intra-molecular configurations with the reciprocal structural relationship of antigen and antibody.* It is possible that the reciprocals exist either within a single molecular species or in distinct substances. For the reactant reciprocal to M, there will be retained the symbol m introduced at the end of step 12 without implying that these sites are identical with the antigenic sites against which the antibodies are directed. Adhesion implies that M and m are each present in the cell surface in such position and arrangement that M and m of one cell can, under proper circumstances, combine with m and M of another cell. If this is so, within the surface of any one cell M and m must be so held in position that they cannot combine with each other.

The structure of a cell surface, with respect to adhesion, would then be crudely analogous to a balloon on which many snap-fastener parts have been attached, male and female separately but interspersed. And the *unit* reaction in adhesions would be



The M-m bonding would be that of antigen to antibody; the  bonding would be whatever holds the components of a cell surface in position.

Is this necessarily a correct picture? Obviously a yes-or-no answer can not be given. At best one might hope to calculate a probability that it is: one would have to estimate the probability of correctness at each step in reaching the final conclusion, and multiply those component probabilities together. Here too, it seems impossible to give a specific answer.

One conclusion of some immunological interest seems inescapable; that, when Microciona and Cliona cells are injected together into a rabbit, some antibody molecules are produced with one or more groups reactive to M or m *and* one or more groups reactive to A or a. These may be termed mixed or heterovalent antibodies. If such antibodies are formed—presumably along with homovalent anti*M*, anti*m*, anti*A*, and anti*a*—two opposing processes will together determine what happens when a mixture of Microciona and Cliona cells is put into anti-Microciona: Cliona serum:

1. blockage of M, m, A, and a, by the homovalent antibodies;

2. linkage of heterovalent antibodies to M or m of a Microciona cell *and* to A or a of a Cliona cell.

Thus of the total antibody combined with M or m, a certain proportion is heterovalent, with a second group that can react with the remaining uncombined A or a. This should lead to aggregation between cells of the two species as described in the fourth paragraph of the discussion.

Examination of standard texts and monographs does not give the non-immunologist any clear impression whether or not it is reasonable to account for the present results as has been done, in terms of heterovalent antibodies. Burnet and Fenner (1950) say (p. 36) "There is fairly conclusive evidence that an antibody cannot function as such against two different antigens," referring to work of Dean, Taylor and Adair (1935).

Roesel (1951), on the other hand, has obtained evidence indicating that heterovalent antibodies are produced in the rabbit in response to injections of a mixture of the bacteriophages T_2 and T_5. Race, Sanger, and Lawler (1948) believe that the majority of anti-C (anti-rh′) sera are really anti-C + C^w in specificity since either antigen, C^w or C, is capable of removing both antibodies. They feel that both components, anti-C and anti-C^w are on the same molecule.

There is therefore some evidence in favor of heterovalent antibodies and the assumption of such antibodies offers a satisfactory explanation of the experimental results obtained with mixed cells in mixed antiserum.

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Summary

1. An investigation of the substances that bind adjacent cell surfaces together is reported here, using two species of sponge, *Microciona prolifera* and *Cliona celata*. Antisera were made, in rabbits, to cell suspensions of each species and to a mixture of cells of both species.

2. Reaggregation of dissociated cells was reversibly inhibited in the homologous antiserum. In normal serum containing cells of both species, the cells sort out to

form aggregates consisting entirely of either one species or of the other, *never* of both species. In an antiserum vs. both species, large aggregates were observed which consisted of cells of both species distributed at random throughout each aggregate.

3. Calcium-free and high calcium media had no effect on reaggregation.

4. The results are compatible with the Tyler-Weiss hypothesis that contiguous cell surfaces are normally held together by forces like those between antigens and homologous antibodies.

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